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Short Communication

Salt-induced Differential Gene Expression in Italian Ryegrass (*Lolium multiflorum* Lam.) Revealed by Annealing Control Primer Based GeneFishing approach

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ABSTRACT

Salt stress is one of the most limiting factors that reduce plant growth, development and yield. However, identification of salt-inducible genes is an initial step for understanding the adaptive response of plants to salt stress. In this study, we used an annealing control primer (ACP) based GeneFishing technique to identify differentially expressed genes (DEGs) in Italian ryegrass seedlings under salt stress. Ten-day-old seedlings were exposed to 100 mM NaCl for 6 h. Using 60 ACPs, a total 8 up-regulated genes were identified and sequenced. We identified several promising genes encoding alpha-glactosidase b, light harvesting chlorophyll a/b binding protein, metallothionein-like protein 3B-like, translation factor SUI, translation initiation factor eIF1, glyceraldehyde-3-phosphate dehydrogenase 2 and elongation factor 1-alpha. These genes were mostly involved in plant development, signaling, ROS detoxification and salt acclimation. However, this study provides new molecular information of several genes to understand the salt stress response. These genes would be useful for the enhancement of salt stress tolerance in plants.

(**Key words** : Italian Ryegrass, Salt Stress, GeneFishing)

Ⅰ. INTRODUCTION

Salt stress is one of the most detrimental factors that reduce plant growth and yield globally. Approximately, 77 million hectors of cultivated land (~ 5% of total land) is greatly affected by salt stress (Munns et al., 1999). A series of physiological, biochemical, molecular and morphological changes is induced by salt stress in plants (Khan et al., 2016). Salt stress induced ionic toxicity in cells is due to presence of high level of Na^+, and Cl^- ions. As a consequence of stress, several free radicals including reactive oxygen species (ROS) may generate in plant cells. ROS induces lipid peroxidations that lead to oxidative stress induced cellular injury in plants (Sharma et al., 2012). However, plants have innate ability to survive salinity stress by using the complex adaptive mechanisms. For instance, plant can tolerate salt stress by maintaining the homeostasis of intercellular ions (Na^+, Cl^-) at plasma membrane by vacuolar compartmentation. In addition, plants have evolved to maintain osmotic potential by cytoplasmic accumulation of osmoprotectants (Diédhiou et al., 2008). Therefore, the identification of salt-stress-induced differentially expressed genes (DEGs) would be potential step for understanding the adaptive response of plants to salinity stress.

Italian ryegrass (*Lolium multiflorum* Lam.) is one of the most cultivated forage crops in Korea, moderately tolerant to salinity. Numerous regulatory proteins/enzymes have been identified in several fodder crops including tall fescue (Martin et al., 2012), perennial ryegrass (Hu et al., 2012), alfalfa (Rahman et al., 2015), and vetiver grass (Liu et al., 2016) which is mediate gene expression under salt stress. Plants respond to salt stress at different stages including physiological and molecular levels. Advance genomic tools provide the clear understanding of global gene expression in plants. Regulation of gene expression, signaling and salt stress tolerance have been demonstrated in model plant *Arabidopsis* (Song et al., 2016), rice (Kawasaki et al., 2001), barley (Hurkman et al.,1996) and soybean (Chen et al., 2014). Despite of great progresses in this field, there are still some molecular issues which need further exploration for Italian ryegrass.

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Several molecular techniques are used to gene expression profiling in plants. For instance, serial analysis of gene expression (SAGE) used to define number and abundance of transcript in plant cells. High-density cDNA microarray approach was applied to understand the benefit of gene function in forage and turf species (Humphreys, 2005). Suppression subtractive hybridization (SSH) is a polymerase chain reaction (PCR) applied to target range of transcript and low abundant genes in plants (Sahu et al., 2012). In recent we are successfully using annealing control primer (ACP)-based GeneFishing technique to identify the DEGs in plants (Rahman et al., 2016b). This ACP-based system increases the annealing specificity to the template and amplifies only the genuine gene products. However, the knowledge about mechanisms of Italian ryegrass responding to salt stress at seedling stage has rarely been reported. Therefore, the objective of this study is to screen salt stress-induced genes in Italian ryegrass seedlings, as well as to identify a set of key genes which played a pivotal under salt tolerance.

Ⅱ. MATERIAL AND METHODS

1. Plant materials and salt treatment

Italian ryegrass (*Lolium multiflorum* Lam. cv. Florida-80) seeds were obtained from Grassland and Forages Division, National Institute of Animal Science (NIAS), Rural Development Administration (RDA), Cheonan, Korea. Italian ryegrass seeds were surface sterilized by 70% ethanol followed by washed three times by Mlli-Q, subsequently treated in 30% sodium hypochlorite (NaOCl) for 30 min, placed on germination medium containing half MS (Murashige & Skoog) and 3% sucrose. Growth chamber temperature was maintained at 25 °C with of 400 μmol m^{-2} s^{-1} light intensity and 14-h/8 h light and dark cycle. One-week old seedlings were exposed to liquid MS medium containing 100 mM NaCl for 6 h, while control plants were treated with normal MS medium. After 6 h of salt treatment the seedlings were washed three times with Milli-Q water, immediately frozen in liquid N2 and preserved at -80 °C until use.

2. Total RNA isolation and first stand cDNA synthesis

Total RNA was isolated from treated and control Italian ryegrass using plant RNeasy mini kt (Qiagen USA). The RNA sample was used for the synthesis of the first stand cDNAs by reverse transcriptase. The reserve transcription reaction was performed for 1.5 h at 42°C using 20 ㎕ containing 3 ㎍ of the purified total RNA, 4 ㎕ of 5x reaction buffer (Promega, USA), 5 ㎕ of dNTPs (2 mmol each); 2 μl of 10 μM dT-ACP1 [5′-CTGTGAATGCTGCGACTACGA TIIIIIT(18)-3′]; 0.5 μl of RNasin RNase Inhibitor (40 U/μl; Promega); and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl; Promega). After completion of first-strand cDNAs synthesis, samples were diluted independently by adding 80 μl of ultra-purified water, and subsequently prepared for GeneFishingTM method.

3. Annealing control primer (ACP)-based GeneFishing™ reverse transcription polymerase chain reaction

An annealing control primer (ACP)‐based PCR technique was performed for isolation of DEGs. In this process gene fishing-kit (Seegene, Korea)was used. Briefly, the second strand cDNA synthesis was conducted using PCR protocol and experimental method of Lee et al. (2011). The amplified PCR products were collected then separated using 1% agarose gel containing 5 μl/100 ml RedSafe™ nucleic acid staining solution (ABC Scientific, USA).

4. Gene cloning and sequence analysis

Salt-induced DEGs were isolated from the gel by using the GENCLEAN II Kit (Q-BIO gene, USA), followed by cloned into a TOPO TA cloning vector (Invitrogen, USA) according to the manufacturer's instruction. The sequences of cloned plasmids were performed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA) using the M13 forward primer (5'-CGCCAGGGTTTTCCCAGTCACGA-3') and M13 reverse primer (5'-AGCGGATAACAATTTCACACAGGA-3'). Finally, the sequences of specific genes were confirmed from NCBI database using Blast tool. (https://blast.ncbi.nlm.nih.gov/Blast)

Ⅲ. RESULTS AND DISCUSSION

In this study, we identified several salt-induced DEGs including alpha-glactosidase b (named as DEG 1), light harvesting chlorophyll a/b binding protein (DEGs 2, and 4), metallothionein-like protein 3B-like (DEG 3), translation factor SUI (DEG5), translation initiation factor eIF1 (DEG 6), glyceraldehyde-3-phosphate dehydrogenase 2 (DEG 7) and elongation factor 1-alpha (DEG 8) in Italian ryegrass seedlings (Fig. 1; Table1). In the following sections, we discussed the potential role of these genes in focus of previous genomics and physiological studies in plant system.

In this study, DEG1 was identified as alpha-glactosidase b (*α*-Gal b) that induced by salt stress in Italian ryegrass seedlings (Fig. 1). Plant *α*-Gals belong to glycoside hydrolase family 27, which have essential role during plant leaf development (Chrost, 2007). In addition, it has been reported that *α-Gal* involved in freezing tolerance in plants (Pennycooke et al., 2003). Here, the up-regulation of α-*Gal* gene indicates that *α-Gal* might be also involved in salt tolerance in Italian ryegrass. Light harvesting chlorophyll a/b binding protein (LHCB; DEGs 2, and 4) is a key apoproteins of the light-harvesting complex of photosystem II (PSII), involved in guard cell signaling suggest that it is possibly involved in salt stress tolerance. Recently, it has been found LHBC gene expression is regulated by several environmental cues and stress signals were induced and transmitted among the plants cells (Liu et al., 2013).

We identified *metallothionein-like protein 3B-like* (*MT3B*; DEG3) that was up-regulated by salt stress. Plant metaloproteins (MTs) belong to cysteine rich protein family. Recently it has been found that the type 1 metallothionein gene is induced by NaCl and CdCl2, involved in detoxification of heavy metal ion, also protected ROS induced cellular injury (Yang et al., 2015). In our study, the up-regulation of *MT3B* gene indicates abiotic stress induced gene that may enhance salt tolerance in Italian ryegrass seedlings. DEG5 was identified as translation factor S*hortened Uppermost Internode* (SUI). The *SUI1*family of genes has important role in stem development of plants (Yin et al., 2013). We speculate that the up-regulation of *SUI* gene involved in cell expansion in Italian ryegrass seedlings. It has been demonstrated that overexpression of *SUI1* and *SUI2* induced outgrowth of internodes during vegetative development in rice (Yin et al., 2013). However, this observation supports to our speculation that provide the role of *SUI* gene family in plants.

A gene (DEG6) encoded translation initiation factor *eIF1*,

Table 1. Salt (NaCl)-stress induced differentially expressed genes (DEGs) in Italian ryegrass seedlings identified by sequence analysis. The sequences were searched by BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/).

DEG No.	ACP	Identity BLAST (blastx)	Total score	E value	Identity	Accession
DEG 1	U5	Alpha-glactosidase b [*Hordeum vulgare*]	358	1.00E-71	87%	Y13848.1
DEG 2	U7	Light harvesting chlorophyll a/b binding protein [*Panicum virgatum*]	512	1.00E-141	87%	JQ425115.1
DEG 3	U8	Metallothionein-like protein 3B-like [*Brachypodium distachyon*]	113	9.00E-22	93%	XM_003565067.1
DEG 4	U16	Light harvesting chlorophyll a/b binding protein [*Zea mays*]	505	2.00E-139	87%	NM_001111957.1
DEG 5	U34	Translation factor SUI(SUI1gene), partial [*Phleum pratense*]	553	7.00E-154	88%	AJ249397.1
DEG 6	U50	Translation initiation factor eIF1 [*Oriza sativa*]	361	4.00E-96	92%	FJ358538.1
DEG 7	U55	Glyceraldehyde-3-phosphate dehydrogenase 2 [*Festuca arundinacea*]	1989	0.0	96%	GQ480773.1
DEG 8	U57	Elongation factor 1-alpha (EF1a) [*Lolium perenne*]	1572	0.0	97%	EU168438.1

played pivotal role in abiotic stress tolerance in rice plants (Rangan et al., 2009). The overexpression of sugar beet *eIFIA* (BveIF1A) improved salt tolerance in model plant *A. thaliana* (Rausell et al., 2003). Moreover, it has been found that the expression of rice *eIF1* (*OseIF1*) gene is induced by ABA, and osmotic stress (Rangan et al., 2009). However, the above discussion supports to up-regulated initiation factor *eIF1* that might be useful for enhancement of salt stress tolerance in plants.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; DEG7) is a ubiquitous enzyme that catalyzes the conversion of glycerate-3-phosphate (3-PGA) to glyceraldehyde-3-phosphate in the presence of ATP and NADPH. In this study, the up-regulation of *glyceraldehyde 3-phosphate dehydrogenase 2* might be involved in salt tolerance in Italian ryegrass. As a similar observation has been documented, genome wide profiling of GAPDH genes revealed that GAPDHs are involved in abiotic stress response (Zeng et al., 2016). However, the expression of several homologs of GAPDHs including gapA/B, gapC, gapCp and gapN indicates that these genes may play pivotal role in abiotic stress tolerance in wheat.

Interestingly, *eukaryotic elongation factor 1 alpha* (eEF1a; DEG8) also identified in Italian ryegrass seedling response to salt stress. *eEF1a* is an important component for protein biosynthesis, and it involved in signal transduction, DNA replication/repair protein networks regulation, molecular chaperone-like activity, and plant development (Suhandono et al., 2014). In this study, we observed that *eEF1a* (DEG7) is up-regulated at seedling stage of Italian ryegrass after salt treatment. It has been previously reported that *eEF1A* genes in cassava (MeEF1A1) were expressed in early stages of plant development, and also eEF1A induced by several environmental stimuli including high temperature, drought and light (Suhandono et al., 2014). Recently, the *eEF1a* was identified in *Urochloa brizantha* (Takamori et al., 2017), elongation factor Tu was regulated by drought stress in alfalfa (Rahman et al., 2016a). However, from the above discussion it suggests that

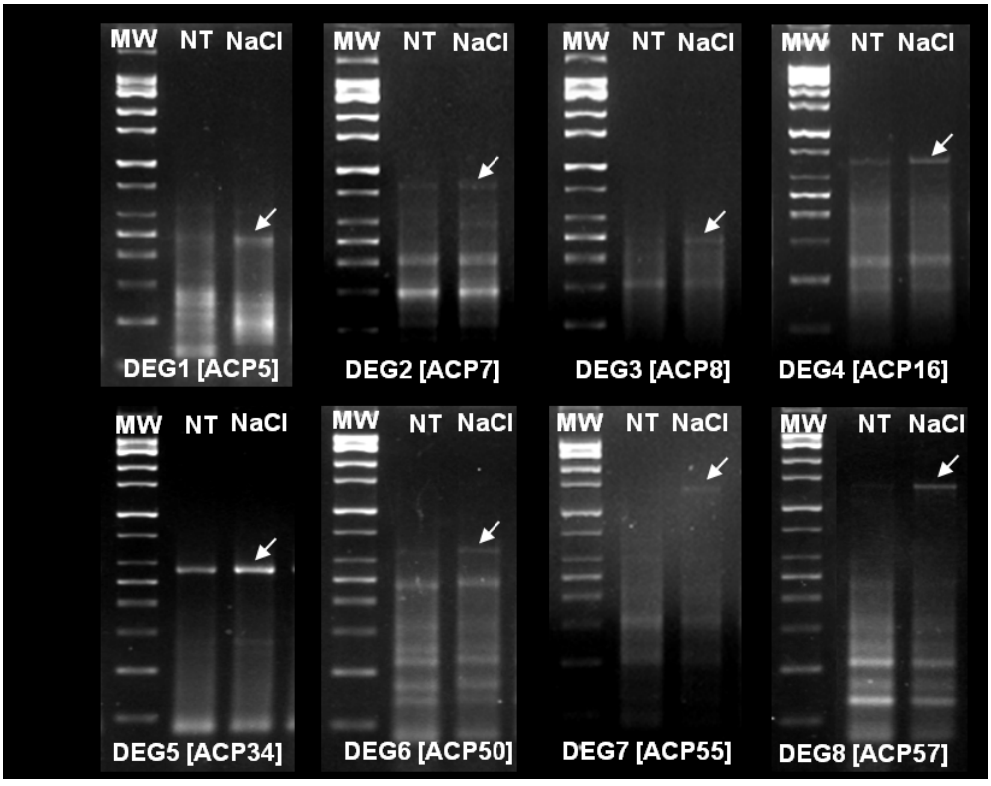

Figure 1. Annealing control primer based GeneFishing approach. Agarose gel image shows differentially expressed genes (DEGs) response to salt (NaCl) stress. Arrows indicate the induced DEGs compared to non-treatment. MW, molecular weight size marker; NT, non-treatment; NaCl, 100 mM salt treatment; ACP, annealing control gene fishing primer.

eEF1A gene has multifunction that can contribute in plant development under external stimuli.

Ⅳ. CONCLUSION

In this study, several differentially expressed genes including alpha-glactosidase b, light harvesting chlorophyll a/b binding protein, metallothionein-like protein 3B-like, translation factor SUI, translation initiation factor eIF1, glyceraldehyde-3-phosphate dehydrogenase 2 and elongation factor 1-alpha were identified using annealing control primer-based GeneFishing-technique. These genes were significantly up-regulated by salt stress in Italian ryegrass seedlings. These genes were involved in plant development, signaling, ROS detoxification and salt acclimation. The identification of these genes would be useful as promising candidates for the salt stress tolerant crop production.

Ⅴ. ACKNOWLEDGMENTS

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